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ANTI-ED-B MONOCLONAL ANTIBODY

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ANTI-ED-B MONOCLONAL ANTIBODY

[Ko ED-B Monoclonal Kotai]

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[There are no amendments to this patent.]

Specification /1*

Title of the invention

Anti-ED-B Monoclonal Antibody

Claims

(1) An anti-ED-B monoclonal antibody characterized by recognizing the amino acid sequence of ED-B represented by the following formula (1).

Glu-Val-Pro-Gln-Leu-Thr-Asp-Leu-Ser-Phe-
Val-Asp-Ile-Thr-Asp-Ser-Ser-Ile-Gly-Leu-
Arg-Trp-Thr-Pro-Leu-Asn-Ser-Ser-Thr-Ile-
Ile-Gly-Tyr-Arg-Ile-Thr-Val-Val-Ala-Ala-
Gly-Glu-Gly-Ile-Pro-Ile-Phe-Glu-Asp-Phe-
Val-Asp-Ser-Ser-Val-Gly-Tyr-Tyr-Thr-Val-
Thr-Gly-Leu-Glu-Pro-Gly-Ile-Asp-Tyr-Asp-
Ile-Ser-Val-Ile-Thr-Leu-Ile-Asn-Gly-Gly-
Glu-Ser-Ala-Pro-Thr-Thr-Leu-Thr-Gln-Gln-
Thr

(2) The anti-ED-B monoclonal antibody of Claim 1 obtainable by using a fused protein of the ED-B region 91 amino acids and protein A as an antigen.

Detailed explanation of the invention

Industrial application field

This invention pertains to an anti-ED-B monoclonal antibody. In particular, it pertains to a novel monoclonal antibody to fibronectin (FN), especially FN of a type contained in cancer tissues.

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* [Numbers in the margin indicate pagination in the foreign text.]

Prior art

FN is first reported by Morrison in 1948 as one of the plasma proteins [Morrison, P.R., et al., J. Am, Chem. Soc., 70, 3103 (1948)], it is a series of multifunctional glycoproteins widely distributed in various tissues and body fluids, and it has been known to be involved in various biological phenomena such as cellular movement, differentiation, proliferation, cancer formation, etc. [Kiyotoshi Sekiguchi, Saibo Kogaku, 4(6), 485-497 (1985)].

It has been known previously that there are two molecular types of FN; FN produced in /2
the liver and present in blood is called plasma FN (pFN), FN on the surface of cultivated cells and in culture solutions is called cellular FN (cFN), and the molecular variety of these kinds of FN has been elucidated to be attributable to alternative splicing of the initial gene transcription products. The sites of such alternate splicing are 3 regions called ED-A, ED-B and IIIcs, and as a result of various combinations of the manifestation of these regions and others, many molecular varieties are considered to be generated.

On the other hand, the FN found in cancer tissues (called cancer FN, below) is an FN having abnormally high manifestation of the above ED-B region, and it has been known as an FN having an ED-B region comprising 91 amino acids [Luciano Zardi, et al., The EMBO Journal, 6 (8), 2337-2342 (1987)].

Objective of the invention

Under such present circumstance as the one described above, it has been desirable to develop means enabling the specific measurement (detection) and purification of the molecular type to carry out studies on the cancer FN described above at the molecular level and eventually enable cancer diagnoses.

The object of this invention is to provide a means coinciding with the above need. Namely, this invention is to provide a monoclonal antibody recognizing ED-B specifically, that is, having reaction specificity to the cancer FN; peptide related to ED-B, especially, specific peptide, which can be an antigen for the production of the above monoclonal antibody and tracer for the cancer FN measurement; and art of the measurement of cancer FN or ED-B in not only the previously used solid phase but also in the liquid phase.

Means to accomplish the objective

According to this invention, an anti-ED-B monoclonal antibody characterized by recognizing the amino acid sequence of ED-B represented by the following formula (1) is provided.

Glu-Val-Pro-Gln-Leu-Thr-Asp-Leu-Ser-Phe-
Val-Asp-Ile-Thr-Asp-Ser-Ser-Ile-Gly-Leu-
Arg-Trp-Thr-Pro-Leu-Asn-Ser-Ser-Thr-Ile-
Ile-Gly-Tyr-Arg-Ile-Thr-Val-Val-Ala-Ala-
Gly-Glu-Gly-Ile-Pro-Ile-Phe-Glu-Asp-Phe-
Val-Asp-Ser-Ser-Val-Gly-Tyr-Tyr-Thr-Val-
Thr-Gly-Leu-Glu-Pro-Gly-Ile-Asp-Tyr-Asp-
Ile-Ser-Val-Ile-Thr-Leu-Ile-Asn-Gly-Gly-
Glu-Ser-Ala-Pro-Thr-Thr-Leu-Thr-Gln-Gln-
Thr

Furthermore, this invention provides a peptide comprising a fused protein of the above 91 amino acids of the ED-B region represented by the above amino acid sequence and protein A.

In the specification described above and below, the IUPAC standards and other common used symbols in the field are used when the amino acids, peptides protective groups, active groups and others are to be abbreviated.

The above specific anti-ED-B monoclonal antibody provided by this invention is an antibody specifically recognizing ED-B and characterized by having a reaction specificity to FN, particularly cancer FN.

Therefore, the antibody of this invention is usable as a specific antibody in the immunoassay of ED-B or cancer FN establishing those highly sensitive, highly accurate and convenient assay methods. Moreover, if the assay methods are established, the arts of cancer screening and diagnoses are provided, and at the same time, they are extremely useful for basic studies such as research, elucidation, etc., of the mechanism of cancer development.

In addition, the antibody of this invention is useful for immunological purification of ED-B or FN, for example, affinity chromatography, etc. /3

In addition, the specific peptide (ED-B-protein A fused peptide) is useful as an immunogen for the production of the anti-ED-B antibody of this invention, and it is also usable as a tracer (label), etc., used in the assay methods described above.

The process for the production of the antibody of this invention is described in detail as follows.

The antibody of this invention can be produced by using fused protein of 91 amino acids of the ED-B region represented by the formula (1) and protein A as an immunogen and conventional production method [Hanfland, P., Chem. Phys. Lipids, 15, 105 (1975); Hanfland,

P., Chem. Phys. Lipids, 10, 201 (1976) sic; Koscielak, J., Eur. J. Biochem. //m., 3//, 37, 214 (1978)].

Incidentally, the ED-B region described above has been known, and its gene has been determined.

Specifically, the above method is carried out by, for example, preparing a fused cell (hybridoma) of a plasma cell of a mammal immunized with the above immunogen (immunized cell) with a tumor cell of the same mammalian plasma cell, selecting a clone producing the desired antibody (monoclonal antibody) recognizing the ED-B region of FN, and cultivating that clone.

The antibody of this invention may be a crude antibody solution, that is, antibody-producing hybridoma culture supernatant and mouse ascites as they are, or their purified forms obtained by carrying out ammonium sulfate fractionation, ion-exchange chromatography, affinity chromatography to use a protein A column, etc.

The fused protein of 91 amino acids of the ED-B region represented by the formula (1) and protein A used as an immunogen in the production of the antibody of this invention is not especially limited as long as it has at least the amino acid sequence represented by the formula (1) and, for example, there are fused proteins of cancer FN prepared from a cancer tissue, ED-B region of that cancer FN or its fragment, synthetic peptide having the above specific amino acid sequence, etc., with protein A. The optimal example among them is the one prepared by using the 91 amino acids of the ED-B region of this invention as a hapten.

The fused protein of 91 amino acids of the ED-B region represented by the formula (1) and protein A described above is preferably prepared by using an established cell strain of a cancer FN having the ED-B region of FN and genetic engineering techniques. The details are as follows.

Specifically, the total RNA is prepared from a cancer FN-producing cultivated established cell strain, for example, typically a WI-38VA13 cell, which is an established cell line prepared by transforming (turning cancerous) a normal diploid fibroblast [transliteration] WI-38 isolated from a human fetal lung tissue with an oncogenic virus SV40 by using the guanidine thiocyanate method (Chirgwin, J.M., et al., Biochemistry, 18, 5294-5299 (1979)). Subsequently, poly(A^+)RNA is selected from the RNA prepared by using an oligo-dT cellulose column, and the cDNA coding the ED-B region is subsequently synthesized by using the polymerase chain reaction method (abbreviated to "PCR method," below, Saiki, R.K., et al., Science, 230, 1350-1354 (1985)) according to the method of Kawasaki and Wang [Kawasaki and Wang, PCR Technology, H.A. Erlich, ed., Stockton Press, New York, 89-98 (1989)]

Namely, a random hexamer is used as a primer to synthesize a single chain cDNA by using reverse transcriptase, and subsequently by using 5'-CAGAGCTCCTGCACTTTTGA-3' as

an upstream primer and 3'-TGTGACTGTGTTGTTTGCC-5' as an downstream primer and the PCR method, the Sac I-Pvu II region coding the ED-B region of FN cDNA can be amplified. The two primer chains used are not necessarily restricted to the above base sequences, and they /4
may be any chains as long as they contain the desired Sac I and Pvu II sites. The double chain cDNA prepared as described above is cut with Sac I and Pvu II, and subsequently together with the Pve II-A I [sic] fragment of FN cDNA cut out from an FN cDNA-containing plasmid [K. Sekiguchi, et al., Biochemistry, 25, 4936-4941 (1986)], it is inserted into the Sac I-Acc I site of plasmid pGEM4 (commercially available from Promega Corp.) to obtain a cDNA clone (pGEMB1) coding the ED-B region of FN and adjoining regions.

Subsequently, the cDNA coding the ED-B-containing region is recovered from the pGEMB1 clone described above as an Eco RI-Pst I fragment and inserted at the Eco RI-Pst I site of the protein A gene fused vector pRIT2T (Pharmacia) to obtain the desired manifestation vector pPAB1 of protein A and ED-B.

The transformation of a host by the manifestation vector prepared as described above is carried out by, for example, using *E. coli* N4830 (purchased from Pharmacia) having λCI_{857} temperature-sensitive repressor as a host and calcium phosphate method [D. Hanahan, D.M. Glover, ed., DNA Cloning, Vol. 1, 109-135, IRL Press, Oxford, 1985]. The transformed bacterium is cultivated in an LB cultivation medium, and subsequently, the cloning procedures of Hanahan and Meselson [Hanahan, D. and Meselson M., Gene, 10, 63-67 (1980)] are carried out to the desired protein A-EB-D fused protein-positive clone.

The production of the desired fused protein can be carried out by carrying out the isolation of the above positive clone, cultivation and heat induction. The protein prepared can be recovered by carrying out an ultrasonic pulverization treatment, thus allowing it to be released from the biomass and purified by using chromatography using an immunoglobulin insolubilized column. As a result, the desired immunogen is prepared.

Incidentally, in the method described above, the ED-B gene is cloned in the pGEM4 vector by dividing to a Sac I-Pvu II fragment and Pvu II-Acc I fragment coding the ED-B region, but it is not necessarily required to do so and, for example, a Sac I-A I fragment may be amplified by the PCR method from the beginning. Furthermore, the above gene may be chemically total-synthesized by using a conventional method such as phosphite triester method [Nature, 310, 105 (1984)], etc.

In the production process of the monoclonal antibody of this invention, the animal to be immunized with the antigen, that is, the above fused protein of protein A and ED-B region is not especially restricted, it is desirably selected by considering the compatibility with the KEISITU [transliteration] cell tumor cell used for cell fusion and, in general, mice, rats, etc., are advantageously used.

The immunization is carried out by using a conventional method, for example, administering the above antigen as it is or after its bonded to a carrier (highly antigenic foreign protein) by using a suitable bonding reagent, described later, via intravenous, muscular, subcutaneous or abdominal cavity injection to a mammal.

As a carrier usable for the above production of an immunological antigen, natural or synthetic polymeric proteins conventionally used for the preparation of antigens are widely usable. Specifically, there are, for example, animal serum albumins such as horse serum albumin, bovine serum albumin, rabbit serum albumin, human serum albumin, sheep serum albumin, etc.; animal serum globulins such as horse serum globulin, bovine serum globulin, rabbit serum globulin, human serum globulin, sheep serum globulin, etc.; animal hemoglobins such as horse hemoglobin, bovine hemoglobin, rabbit hemoglobin, human hemoglobin, sheep hemoglobin, etc.; animal hemocyanins such as keyhole limpet hemocyanin (KLH), etc.; proteins extracted from roundworm [*Ascaris* extracts, Japanese Kokai Patent Application Publication No. Sho 56[1981]-16 414, J. Immun., 111, 260-268 (1973); J. Immun., 122, 302-308 (1979); J. Immun., 98, 893-900 (1967) and Am. J. Physiol., 199, 575-578 (1960), their purified products]; and polymers such as polylysine, polyglutamic acid, lysine-glutamic acid copolymer, other copolymers containing lysine or ortinine, etc. /5

As a hapten-carrier binding reagent, any of those widely used for antigen preparation may be used. Specifically, diazonium compounds such as bisdiazotized benzidine (BDB), bisdiazotized 3,3'-dianisidine (BDD), etc., allowing tyrosine, histidine and tryptophan to form cross-linking bonding; aliphatic dialdehydes such as glyoxal, malondialdehyde, glutaraldehyde, succinaldehyde, adipoaldehyde, etc., cross-linking amino groups; dimaleimides such as N,N'-o-phenylene dimaleimide, N,N'-m-phenylene dimaleimide, etc., cross-linking thiol groups; maleimidecarboxyl-N-hydroxysuccimide esters such as metamaleimidebenzoyl-N-hydroxysuccimide ester, 4-(maleimidemethyl)-cyclohexane-1-carboxyl-N'-hydroxysuccimide ester, N-hydroxysuccimidyl-3-(2-pyridyldicyclo) propionate (SPDP), etc., cross-linking amino and thiol groups; and reagents used for conventional peptide bonding formation reactions forming amide bonding of amino and carboxyl groups, for example, dehydration-condensation agents such as N,N-dicyclohexylcarbodiimide (DCC), N-ethyl-N'-dimethylaminocarbodiimide, 1-ethyl-3-diisopropylaminocarbodiimide, 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide, etc. Furthermore, it is also possible to use the combinations of diazonium-allylcarboxylic acids such as p-diazoniumphenylacetic acid, etc., with peptide bond-forming reaction reagents such as the above dehydration-condensation agents, etc., as a hapten-carrier binding agent.

The above process for the production of an immunological antigen by using a hapten, carrier protein, hapten-carrier binding reagent, spacer, etc., can be carried out by using conventional procedures. In general, the reaction is carried out in a aqueous solution or buffer

solution at pH 5-10, preferably pH 6-9 at 0-40°C, preferably around room temperature. The reaction can be completed generally in 2-5 h.

The amounts of the hapten, hapten-carrier binding reagent, and carrier of the above reaction are suitably determined, but in general, the amount of the carrier is about 0.5-5 times, preferably 1-2 times, the weight of the hapten used. The amount of the hapten-carrier binding reagent to be used is 1-30 times the amount of mole of the hapten used. By carrying out the above reaction, the desired hapten-carrier complex bonded directly or bonded through a spacer is obtained.

The antigen obtained after the reaction is easily isolated and purified by conventional procedures such as dialysis, gel filtration, fractionation precipitation, etc.

More specifically, the immunization described above can be carried out by diluting the immunogen to a suitable concentration with a physiologically saline solution-containing phosphate buffer solution (PBS), if necessary, using a conventionally used adjuvant, administering to an animal several times every 2-14 days and setting the total dose in the range of about 10-100 μg in the case of mice or about 0.2-2.0 mg in the case of rabbits. As an adjuvant, whooping cough vaccine, complete Freund's adjuvant and alum, etc., are usable.

The antibody produced is harvested by collecting blood from the immunized animal after /6
1-2 wereks from the final administration, and the blood collected is centrifuged to obtain serum.

As an immunized cell used for the monoclonal antibody production, a spleen cell extracted about 3 days after the above final administration is preferably used.

As a mammal plasmacytoma cell used as the other parent cell to be fused with the above immunized cell, there are various known usable cells such as p3/x63-Ag8 (X63) [Nature, 256, 495-497 (1975)], p3/x63-Ag8. U1 (P3U1) [Current Topics in Microbiology and Immunology, 81, 1-7 (1978)], P3/NSI-1-Ag4-1 (NS-1) [Eur. J. Immunol., 6, 511-519 (1976)], Sp2/0-Ag14 (Sp2/0) [Nature, 276, 269-270 (1978)], FO [J. Immunol. Meth., 35, 1-21 (1980)], etc., and myeloma cells in rats such as 210, RCY3, Ag1, 2, 3, (Y3), etc., [Nature, 277, 131 (1979)].

The reaction for fusion betweren the above immunized cell and plasmacytoma cell is carried out by using any of known methods such as the method of Milstein, et al. [Method in Enzymology, 73, 3 (1981)], etc. Specifically, the above fusion reaction is carried out in a conventional cultivation medium in the presence of a conventional fusion promoter such as polyethylene glycol (PEG), Sendai virus (HVJ), etc., and if necessary, the cultivation medium may contain an auxiliary component such as dimethylsulfoxide, etc., to promote the fusion efficiency further. Moreover, an electric treatment (electric fusion) may be suitable. The ratio of the immunized cell and plasmacytoma cell is same as that in any other method of fusion reactions, for example, the amount of the immunized cell is about 1-10 times the amount of the plasmacytoma cell. As a cultivation medium used in the fusion reaction, any of various

cultivation media usable for proliferation of the above plasmacytoma cell such as RPMI-1640 medium, MEM medium and other conventionally used media, and from those media, serum auxiliary components such as fetal cow serum (FCS), etc., are preferably removed.

The cell fusion reaction is carried out by thoroughly mixing required amounts of the above immunized cell and plasmacytoma cell in the above cultivation medium, adding and mixing generally about 30-60 w/v% to the cultivation medium of PEG with a molecular weight of, for example, 1000-6000 heated to about 37°C in advance. Subsequently, the medium is suitably supplemented, the culture is centrifuged to remove the supernatant, and the procedures are repeated to prepare the desired hybridoma.

The isolation of the desired hybridoma prepared is generally carried out by cultivating in a selection cultivation medium such as HAT medium (cultivation medium containing hypoxanthine, aminopterin and thymidine). This cultivation in the HAT medium may be carried out for the length of time sufficient for the cells (cells not used for the fusion reaction, etc.), other than the hybridoma to die, generally several days to several wereks. The hybridoma prepared is used for testing the desired antibody and single cloning by using the conventional limiting dilution method.

For testing of a desired antibody-producing strain, for example, various methods [Hybridoma method and monoclonal antibody, R & D Plannng K.K., 30-53, March 5, 1982] such as the ELISA method [Engvall, E., Meth. Enzymol., 70, 419-439 (1980)], plaque method, spot method, agglutination reaction method, Ouchterlony method, radioimmunoassay (RIA) method, etc., are usable, and for this searching, the immunological antigen described above is usable.

The hybridoma producing the desired monoclonal antibody of this invention prepared as described above can be successively cultivated in a conventional cultivation medium or stored in liquid nitrogen for a long period of time.

To collect the monoclonal antibody of this invention from the hybridoma prepared as described above, there are various usable methods such as cultivating the hybridoma by using /7
conventional procedures and obtaining the monoclonal antibody in the culture supernatant, administering the hybridoma in a compatible mammal for proliferation and collecting the monoclonal antibody in the ascitic fluid, etc. The former method is suitable for obtaining the monoclonal antibody in a highly pure form, and the latter method is suitable for manufacturing the monoclonal antibody.

The antibody prepared as described above can be purified further by using conventional procedures such as salting out, gel filtration, affinity chromatography, etc.

As described above, the anti-ED-B monoclonal antibody of this invention can be prepared.

The applications of the antibody of this invention are explained in detail as follows. The antibody may be used in conventional purification means such as immunosedimentation, affinity chromatography, etc., to purify the ED-B region of FN conveniently and specifically. Moreover, according to the applications of the antibody of this invention, the immunological reaction may be used to measure cancer FN specifically in a sample such as body fluid, etc. Specific methods include various conventional immunological means such as competitive assay, radioimmunoassay (RIA) by the sandwich method, enzyme-linked immunosorbent assay (ELISA), aggregation assay, agglutination method, etc., and those conventionally used procedures, operations, etc., may be used for those methods and assay means.

Specifically in the case of competitive assay, the competitive reaction of any cancer FN in a sample to be measured and constant amount of ED-B in inactivated FN is carried out with a constant amount of the antibody of this invention labeled with a labeling agent, the complex of the labeled antibody and ED-B of insolubilized FN and nonbound labeled antibody are isolated, and the label activity of one of them is measured to carry out quantitative determination of cancer FN in the sample. If the sandwich method may be carried out as follows. The reaction of a substance to be measured (sample) with the insolubilized antibody of this invention is carried out forming an complex of the insolubilized antibody and ED-B of FN, the complex formed is allowed to react with a constant amount of the labeled antibody and subsequently, the label activity of a bound form of the labeled antibody and the complex formed of nonbound label activity of is measured to carry out quantitative determination of cancer FN in the sample similarly to the method described above.

As a sample usable in the case of those various assay means and methods, there are body fluids such as blood, urine, cellular tissue fluid, etc., and among them, the use of blood, especially serum or plasma, is preferable.

The preparation of the antibody of this invention labeled with a labeling agent and labeled antibody can be carried out by using a suitable labeling agent and conventional procedures. As a labeling agent, there are conventional agents, for example, radioactive substances such as ^{125}I, ^{131}I, tritium, etc., and various enzymatic reagents such as glucoamylase, peroxidase (POX), chymotrypsinogen, procarboxypeptidase,, glyceroalhydyde-3-phosphate dehydrogenase, amylase, phosphorylase, alkali phosphatase, D-Nase, P-Nase, β-galactosidase, glucose-6-phosphate dehydrogenase, ortininedecarboxylase, etc. As a labeling method, for example in the case of radioactive iodine, the oxidative iodization to use chloramine T (refer to W.M. Hunter and F.C. Greenwood, Nature, 194, 495 (1962); Biochem. J., 89, 144 (1963)] may be carried out. The introduction of an enzymatic reagent can be carried out by using a conventional coupling method such as the method of Erlanger (B.F. Erlanger), et al. [Acta.

Endocrinol. Suppl., 168, 206 (1972)] or the method of Karol (M.H. Karol), et al. [Proc. Natl. Acad. Sci., USA, 57, 713 (1967)], etc.

Furthermore, the insolubilized antibody of this invention and insolubilized ED-B of insolubilized FN, for example, those solidified physically or chemically on plates can be prepared by allowing the antibody of this invention or ED-B to bond physically or chemically to a suitable insoluble carrier. As a carrier usable in this case, there are, for example, cellulose powder, Sephadex, Sepharose, polystyrene, filter paper, carboxymethylcellulose, ion-exchange /8
resin, dextran, plastic film, plastic tube, nylon, glad beads, silk, polyamine-ethyl vinyl ether-maleic acid copolymer, amino acid copolymer, ethylene-maleic acid copolymer, etc. The above insolubilization can be carried out by a covalent bonding method such as diazo method, peptide method, alkylation, carrier bonding method using a cross-linking reagent (glutaraldehyde, hexamethylne isocyanate, etc., used as a cross-linking reagent), carrier bonding method using UgI reaction and other chemical reactions, ionic bonding method such as method using ion-exchange resin as a carrier, etc., and physical adsorption method using a porous glass carrier such as glass beads, etc.

The reaction used in the above assay methods (immunological reaction) is carried out at a temperature generally below 45°C, preferably in the range of 4-40°C for several hours up to 24 hours.

As described above, by using the antibody of this invention, it is possible to measure cancer FN or ED-B-containing FN in a sample conveniently and highly accurately.

The modifications and applications of such purification and measurement systems using the antibody of this invention as well as setup and installation of them are apparent to those who in the industry.

Effect of the invention

This invention provides a monoclonal antibody to ED-B of FN and ED-B of FN-protein A fused protein as an antigen for the production of the monoclonal antibody. By utilizing the antibody of this invention, the cancer FN research method and cancer diagnostic and treatment methods are provided.

Application Example

This invention is explained in further detail by using application examples as follows, but this invention is not necessarily limited to those examples.

Application Example 1

Preparation of ED-B-protein A fused protein

(1) Preparation of Sac I-Pvu II fragment containing the ED-B region of FN

a) Cell cultivation

In this example, WI-38VA13 cell was used. The cell is an established cell line prepared by transforming a normal diploid fibroblast WI-38 isolated from a human fetal lung tissue with an oncogenic virus SV40, the properties of which have been elucidated by Girardi (A.J. Girardi), et al. [Ann. Med. Exp. Biol. Fenn., 44, 242-254 (1966)], and it has been deposited at ATCC as ATCC CCL 75.1.

The above WI-38VA13 cell was cultivated according to the method described in R.I. Freshny, "Culture of Animal Cells" (Anan R Lis, Inc., New York, 1983).

The trypsin-treated WI38VA14 cell was sown in 10 dishes of 15 cm cultivation dish (Falcon tissue culture dish #3025) at a density of about 10^6 cells per dish and cultivated in a 10% FCS (fetal cow serum)-containing DME cultivation medium (Dulbecco's modified Eagle cultivation medium, Gibco) in the presence of 5% CO_2 at 37°C for 5 days. Subsequently, the cell was peeled off from the culture dishes by using a rubber policeman, and the centrifugation (500xg for 5 min) was carried out to recover about 1 g of the WI-38VA13 cell.

b) Preparation of cDNA library

About 1 g of the cell prepared in the above section a) was added to a Potter homogenizer containing 15 mL of a homogenization buffer solution [5.3 M guanidinium thiocyanate, 0.02 M N-laurylsarcosyl sodium, 0.03 M trisodium citrate, 0.8% β-mercaptoethanol and 0.7% Antifoam 289 (defoaming agent, Sigma)]. After 10 repetitions, the content was transferred to a beaker, and shearing was carried out by allowing it to pass through a 22G needle attached to a 20 mL cylinder 3 times with great force.

About 4 mL of 5.7 M cesium chloride and 0.1 M EDTA were placed in a centrifugation tube, about 8 mL of the above homogenate was layered over, and centrifugation was carried out at 20°C and 32,000 rpm for 20 h to recover total RNA. /9

The total RNA recovered was diluted to 5 mg/mL or more, and after incubation at 65°C for 7 min, it was cooled with ice for 2 min. The same amount of a 2-times oligo-dT bonded buffer solution (1.0 M NaCl, 20 mM Tris-HCl, pH 7.5) and $1/100^{th}$ volume of 20% SDS were added, and the mixture was mixed thoroughly. Subsequently, it was applied on an oligo-dT cellulose column (BioRad) equilibrated with an oligo-dT bonded buffer solution (0.5 M NaCl, 10 mM Tris-HCl, pH 7.5, 0.1% SDS). The unabsorbed fraction was used to carry out the reaction again at 65°C for 7 min, and after cooling with ice for 2 min, it was applied to the column again. The column was washed with 10 times volume of the oligo-dT bonded buffer solution, and furthermore, the column was washed with a 10 times volume of an oligo-dT washing solution (0.1 M NaCl, 10 mM Tris-HCl, pH 7.5, 0.1% SDS). The A^+RNA bonded to the oligo-dT

cellulose was eluted with an oligo-dT elution solution (10 mM Tris-HCl, pH 7.5, 0.05% SDS). To the eluate, 1/25th volume of 5 M NaCl and 2.5 times volume of ethanol were added, mixed thoroughly and allowed to stand at –20°C for one whole day. The solution was centrifuged at 12,000 rpm for 15 min to allow poly-A$^+$RNA to precipitate, and after suspending in 70% ethanol, it was similarly centrifuged, the precipitates were dried and, subsequently, a suitable amount was dissolved in water.

The region of cDNA of FN coding he ED-B region was amplified from the poly-A$^+$RNA prepared as described above by using the polymerase chain reaction method according to the method of Kawasaki and Wang [Kawasaki and Wang, PCR Technology, H.A. Erlich, ed., Stockton Press, New York, 89-98 (1989)].

c) Primer synthesis

Subsequently 2 oligo-deoxynucleotide primers were prepared.

Upstream primer (Sac I site):

5'-CAGAGCTCCTGCACTTTTGA-3'

Downstream primer (Pvu II site):

3'-TGTGACTGTGTTGTTTGCC-5'

The above primers were prepared as follows. The solid phase method was carried out for syntheses using an automated DNA synthesizer (Applied Biosystems, Model 380A) and β-cyanoethylphosphoamidate derivatives of 4 kinds of bases. The deprotection and separation from the solid-phase carrier of the oligonucleotides synthesized were carried out by heating in concentrated aqueous ammonia at 55°C for 10 h. The synthetic oligonucleotide obtained were purified by HPLC and, finally, about 50 μg each of the desired oligonucleotides were obtained as upstream and downstream primers, respectively.

The purified oligonucleotides prepared were dissolved in a TE buffer solution (10 mM Tris-HCl, pH 7.4, 1 mM EDTA), and the respective solutions were stored at –20°C.

d) Single chain cDNA synthesis

In a 0.5 mL tube (Eppendorf), 10 μL of a 2x reaction buffer solution [40 mM Tris-HCl, pH 8.4, 100 mM KCl, 5 mM $MgCl_2$, 0.2 mg/mL nuclease-free bovine serum albumin, 2 mM dATP, 2 mM dGTP, 2 mM dCTP, 2 mM TTP, 2 units/mL RNasin (Promega) and 100 pmole random hexamer (Pharmacia)] and 9 μL of a solution containing about 1 μg of RNA thermally treated at 90°C for 5 min in advance were added and mixed. Subsequently, 1 μL of mouse Molony leukemia virus-origin reverse transcriptase (about 200 units) was added, and the mixture

was incubated at room temperature for 10 min and at 42°C for 30 min to synthesize a single chain cDNA. The reaction mixture was heated at 95°C for 10 min to stop the reaction.

e) Amplification of Sac I-Pvu II fragment

To 20 μL of the single chain cDNA solution, the reaction of which was stopped by heating in the above section d), 80 μL of a 1x PCR reaction buffer solution (20 mM Tris-HCl, pH 8.4, 4.50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mg/mL nuclease-free bovine serum albumin) containing 50 pmole each of the upstream and downstream primers and 5 units of Taq polymerase (Perkin-Elmer/Cetus, 1 μL) were added, 100 μL of mineral oil was added to layer over, and the procedures of heating at 95°C for 1.5 min, 50°C for 3 min and 72°C for 3 min were repeated 35 times to amplify the Sac I-Pvu II cDNA fragment coding the desired ED-B region. After completing the reaction, 10 units of Sac I was added, and the mixture was incubated at 37°C for 2 h exposing the 5' side Sac I site of the Sac I-Pvu II cDNA fragment amplified. /10

The electrophoresis of the above reaction mixture was carried out in the presence of etidium bromide by using 1.5% agarose gel and Hae III digested DNA fragments of ϕx174 DNA as a molecular weight marker confirming that the desired Sac I-Pvu II fragment of 385 base pairs had been amplified.

f) Purification of Sac I-Pvu II fragment

The Sac I-Pvu II fragment isolated on an agarose gel according to the procedures in the above section e) was transferred and adsorbed on a DEAE cellulose membrane (S and S Co., NA45) by using the method of Dretzen [Dretzen, G.M., Anal. Biochem., 112, 295-298 (1981)]. The adsorbed DNA was eluted from the DEAE cellulose membrane with an elution buffer solution (50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 10 mM EDTA), subsequently, the cold ethanol precipitation was carried out to recover the desired Sac I-Pvu II fragment (about 100 ng).

(2) Preparation of FNcDNA Pvu II-Acc I fragment

a) In 50 μL of a reaction buffer solution (10 mM Tris-HCl, pH 7.5, 5.7 mM $MgCl_2$, 60 mM NaCl, 7 mM 2-mercaptoethanol and 0.01% bovine serum albumin), 20 μg of human fibronectin cDNA clone pLF5 isolated by Sekiguchi, et al. [Sekiguchi, K., et al., Biochemistry, 25, 4936-4941 (1986)] was dissolved, 20 units each of Pvu II and Acc I (Takara Shuzo) were added, and the reaction was carried out at 37°C for 2 h. After completing the reaction, the electrophoresis using a 1% agarose gel was carried out to isolate the desired Pvu II-Acc I fragment (226 base pairs). Subsequently, a DEAE cellulose membrane was used to recover the desired DNA fragment (about 500 ng) by using similar procedures as those in the section (1)f).

(3) Cloning of FN cDNA Sac I-Acc I fragment to pGEM4

In 20 μL of a reaction buffer solution (10 mM Tris-HCl, pH 7.5, 5.7 mM $MgCl_2$, 60 mM NaCl, 7 mM 2-mercaptoethanol and 0.01% bovine serum albumin), 5 μg of pGEM4 (Promega) was dissolved, 10 units of Sac I (Takara Shuzo) and 10 units of Acc I (Takara Shuzo) were added, and the mixture was incubated at 37°C for 2 h to allow the polylinker region of pGEM4 to be cleaved at the Sac I and Acc I sites. After a treatment with phenol, the ethanol precipitation of the reaction product mixture was carried out to recover cleaved plasmid DNA, which was dissolved in 48 μL of reaction buffer solution (50 mM Tris-HCl, pH 9.0, 0.1 mM $ZuCl_2$ [sic], 1 mM $MgCl_2$ and 1 mM spermidine), 20 units of bovine small intestine alkali phosphatase (Takara Shuzo) was subsequently added, and the mixture was heated at 37°C for 15 min and 56°C for 15 min to carry out dephosphorylation of the 5' terminal.

After adding 2.5 μL of 10% SDS, the mixture was heated at 68°C for 15 min to deactivate the enzyme, and after a phenol treatment, the ethanol precipitation was carried out to recover the 5' terminal dephosphorylated plasmid DNA. /11

Subsequently, 20 ng of the above plasmid DNA and 20 ng each of the cDNA fragments prepared in the above sections (1) and (2) were dissolved in 24 μL of a ligation buffer solution (66 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM dithiothreitol and 1 mM ATP), 300 units of T4DNA ligase (Takara Shuzo) were added, and the mixture was incubated at 16°C for 16 h to insert the cDNA fragment from the Sac I site to the Acc I site coding the ED-B region of FN to the Sac I-Acc I site of pGEM4.

Subsequently, 1 μL of the reaction mixture was taken, mixed with 100 μL of *E. coli* HB101 competent cell (Takara Shuzo), and the mixture was incubated for 30 min while cooling in an ice bath and subsequently at 42°C for 90 sec to introduce the plasmid DNA to the *E. coli*. Subsequently, 1 mL of an LB cultivation medium (1% Bacto trypsin, 0.5% yeast extract and 1% table salt) was added, and the shaking cultivation was carried out at 37°C for 1 h. A 50 μg/mL ampicillin-containing LB agar cultivation medium (1.5% Bacto agar, 1% Bacto trypsin, 0.5% yeast extract and 1% table salt) was inoculated with 100 μL of the above shake culture, and the incubation was carried out at 37° for 14 h to obtain about 200 colonies of *E. coli* transformed with the plasmid DNA. Among them, 12 colonies were randomly selected, cultivated in a 50 μg/mL ampicillin-containing LB cultivation medium, and the plasmid DNA was recovered from each colony by using the modified method of Birnbim and Doly [Molecular Cloning: A Laboratory Manual, T. Maniatis, et al., ed., 368-369 (1982)]. By carrying out double digestion with Eco RI and Pst I, a plasmid clone (pGEMB1) having a predicted insertion sequence of about 600 base pairs was selected.

(4) Recovery of Eco RI-Pst I fragment from pGEMB1

a) Plasmid DNA isolation

The *E. coli* strain containing the plasmid clone pGEMB1 prepared in the above section (3) was cultivated at 37°C for 12 h by using a 50 μg/mL ampicillin-containing LB cultivation medium. Subsequently, the biomass was recovered by centrifugation at 5000 G for 10 min, and the alkali bacteriolysis method [Molecular Cloning: A Laboratory Manual, T. Maniatis, et al., ed., 368-369 (1982)] was carried out to isolate the plasmid DNA as follows.

Specifically, the biomass was suspended in 8 mL of a 5 mg/mL lysozyme-containing buffer solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0 and 10 mM EDTA), and the suspension was allowed to stand at room temperature for 5 min. Subsequently, 16 mL of 0.2 N NaOH/1% SDS solution was added, mixed quickly, and the bacteriolysis was carried out while cooling over an ice bath for 10 min. Subsequently, 12 mL of an ice-cooled 5 M potassium acetate solution (pH 4.8) was added, mixed, and the mixture was allowed to stand for 10 min while cooling over an ice bath.

The mixture was subsequently centrifuged at 4°C and 20,000 rpm for 20 min, 32 mL of the supernatant in 16 mL each was placed in 2 Korex glass centrifugation tubes, 10 mL each of isopropanol was added to the respective tubes, and the mixture was allowed to stand at room temperature for 15 min. Subsequently, the mixture was centrifuged at 15°C and 12,000 G for 30 min to recover the plasmid DNA as a precipitate.

The precipitate obtained was dried in air, dissolved in 8 mL of a TE buffer solution (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA), 8g of cesium chloride and 0.4 mL of 1 mg/mL solution of etidium bromide were added, and after mixing thoroughly, the mixture was centrifuged at room temperature and 2000 rpm for 5 min to remove any insoluble inclusions. The supernatant was transferred to a 12 PA seal tube (Hitachi Kogyo), the top portion of the tube was filled with mineral oil, and the centrifugation was carried out at 19°C and 55,000 rpm for 16 h to allow a plasmid DNA band to form. Subsequently, a syringe with a needle was used to recover /12
the plasmid DNA, and the ethanol precipitation was carried out to obtain the desired pGEMB1 plasmid DNA (about 200 μg)

b) Recovery of Eco RI-Pst I fragment

In 25 μL of an Eco RI-Pst I reaction buffer solution (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl and 1 mM dithiothreitol), 5 μg of the pGEMB1 plasmid DNA prepared as described above in the section a) was dissolved, 10 units of Eco RI and 10 units of Pst I were added, and the mixture was incubated at 37°C for 2 h to carry out scission of the plasma DNA at the Eco RI site and Pst I site. For the reaction mixture obtained, 1.5% agarose gel electrophoresis was carried out to isolate the desired Eco RI-Pst I fragment, and the method using a DEAE

cellulose membrane shown in the above section (1) was carried out to recover the desired DNA fragment (about 300 ng).

(5) Insertion of Eco RI-Pst I fragment to pRIT2T

a) Plasmid vector preparation

In 20 μL of an Eco RI-Pst I reaction buffer solution, 2 μg of protein A gene-fused vector pRIT2T (Pharmacia) was dissolved, 10 units each of Eco RI and Pst I were added, and the mixture was incubated at 37°C for 2 h to allow the plasmid DNA for cleavage at the Eco RI-Pst I fragment sites. After phenol extraction of the reaction mixture, the ethanol precipitation was carried out to recover the cleaved plasmid DNA. The method used in section (3) described above was also used to carry out dephosphorylation of the 5' terminal by using bovine small intestine alkali phosphatase. The phenol extraction was carried out again, and the ethanol precipitation was carried out to obtain 1 μg of the desired plasmid vector.

b) Insertion of Eco RI-Pst I fragment to plasmid vector

In 24 μL of the same ligation buffer solution as that described in the above section (3), 20 ng of pRIT2T plasmid cleaved with Eco RI and Pst I with its 5' terminal dephosphorylated as described in a) and 20 ng of the Eco RI-Pst I fragment of pGEMB1-origin prepared in section (4) were dissolved, 300 units of T4DNA ligase was added, and the mixture was incubated at 16°C for 16 h to insert the pGEMB1-origin Eco RI-Pst I fragment into the polylinker region of pRIT2T.

c) Transformed cell preparation

E. coli HB101 strain was transformed by using 1 μL of the reaction solution prepared in the above section b) and method described in the above section (3), and about 50 colonies were obtained on a 9 cm LB agar plate. Among them, 12 colonies were randomly collected, 1.5 mL of LB medium containing ampicillin were added, the modified method of Birnbim and Doly was carried out to recover the plasmid DNA from each colony. To the plasmid DNA (about 1 μg) dissolved in 10 μL of the Eco RI-Pst I reaction buffer solution, 5 units each of Eco RI and Pst I were added, and the mixture was incubated at 37°C for 2 h to obtain a reaction product mixture, which was analyzed by a 1% agarose gel electrophoresis to identify a clone (pPAB1) with the Eco RI-Pst I fragment of 623 base pairs formed.

d) The *E. coli* strain having the plasmid pPAB1 identified in the above section c) was cultivated in a 500 mL ampicillin-containing LB medium, and the same alkali bacteriolysis

method described in section (4a) was carried out to obtain about 300 μg of the desired plasmid DNA pPAB1.

(6) Introduction of plasmid pPAB1 to *E. coli* N4830

The pPAB1 plasmid DNA prepared in the above section (5) was inserted in *E. coli* N4830 (Pharmacia) by using the calcium phosphate method of Mandel and Higa [J. Mol. Biol., 53, 154 (1970)] as follows.

Specifically, the shaking cultivation of *E. coli* N4830 in 100 mL of an LB cultivation medium was carried out, and when the biomass density was about 5 x 10^7/mL, the cultivation was terminated, and the culture was cooled in an ice bath. The biomass was collected by /13
centrifugation at 4°C and 4000xg for 5 min, the precipitate was suspended in an ice-cooled 50 mM calcium chloride-10 mM Tris-HCl (pH 8.0), and the suspension was allowed to stand in an ice bath for 15 min. Subsequently, the centrifugation was carried out at 4°C and 4000 G for 5 min. The precipitate obtained was suspended again in 50 mM calcium chloride-10 mM Tris-HCl (pH 8.0), and the suspension was allowed to stand in an ice bath. To 0.2 mL of the suspension of *E. coli* prepared as described above, 10 μL of a pPAB1 plasmid solution dissolved in a TE buffer solution (containing 10 ng of the plasmid DNA) was added, the mixture was allowed to stand in an ice bath for 30 min, subsequently, it was heated in a warm water bath at 42°C for 2 min, 1 mL of an LB medium was added, and the mixture was incubated at 37°C for 1 h. The ampicillin-containing LB agar medium having the same composition as that described above was inoculated with 100 μL of the *E. coli* suspension, and the incubation was carried out at 37°C for 14 h to form colonies of transformed *E. coli* on the agar cultivation medium.

(7) Isolation of ED-B protein A fusion protein

The shaking cultivation of the transformant (*E. coli* N4830 transformed with the plasmid pPAB1) obtained in the above section (6) was carried out at 30°C in 500 mL of LB medium for 14 h. Subsequently, 500 mL of LB medium heated at 54°C in advance was added, furthermore, the shaking cultivation in a warm water bath at 42°C was carried out for 90 min to induce the manifestation of the ED-B protein A fusion protein.

Subsequently, the biomass was recovered by carrying out centrifugation at 4°C and 5000 G for 15 min, suspended in 100 mL of an ice-cooled tris-buffered physiologically saline solution (50 mM Tris-HCl, pH 7.6, 150 mM NaCl), the ultrasonic pulverization treatment (Branson Sonifier 250, output set at 7, and 3-min treatment repeated 3 times) of the suspension was carried out while cooling over an ice bath, and as a result, the proteins inside the bacterium were released. Subsequently, about 100 μL of the pulverized liquid was centrifuged (16,000 G, 20 min, 4°C) to recover about 95 mL of supernatant, which was diluted with 300 mL of the Tris-

buffered physiological saline solution, and the diluted solution was applied to a column packed with about 10 mL of IgG-Sepharose 6 fast flow (Pharmacia) allowing the ED-B protein A fusion protein to be adsorbed on the column. After washing the column with 100 mL of the tris-buffered physiological saline solution and subsequently 20 mL of 5 mM ammonium acetate solution (pH 5.0), respectively, the protein adsorbed was eluted with a 0.5 M solution of acetic acid. The ED-B protein A fusion protein obtained was dialyzed against the Tris-buffered physiological saline solution for 2 whole days to obtain about 1 mg of the desired antigen.

Application Example 2

Preparation of hybridoma

After dilution with 0.5 mL of PBS, 0.05 mg of the purified ED-B-protein A fusion protein prepared in Application Example 1 was mixed with the same amount of complete Freund's adjuvant, emulsified and 0.2 mL each was administered to male Balb/c mice (8 wereks old) by intradermal injection. Subsequently, the additional administration was carried out 4 times with an interval of 2 wereks for immunization, and on the third day after the final immunization, the spleen was extracted.

From the spleen extracted, the cells were taken out, red corpuscles inside the cells were treated with a 0.83% solution of ammonium chloride at 4°C for 1-2 min and removed by lysis. The cells obtained after the above procedures were collected as sensitized lymphocyte cells and washed 3 times with an RPMI-1640 cultivation medium heated at 37°C.

Subsequently, a mouse myeloma cell [P3U1, see Current Topics in Microbiology and Immunology, 73, 3 (1981)] was successively cultivated in a 15% FCS (fetal cow serum)-containing RPMI-1640 cultivation medium with 100 μM of 8-azaguanine added, the cell prepared was washed and used as a myeloma cell.

The myeloma cell and bone marrow tumor cell [sic] were mixed in a 50 mL tube in the proportion of the number of cells of 10:1, the cell mixture obtained was centrifuged at 500 G for 5 min, and the supernatant was completely removed by using a Pasteur pipette. Those procedures were carried out inside a water tank maintained at 37°C. /14

Subsequently, 4 mL of 35% polyethylene glycol 1500 (Wako Junyaku, called PEG, below) was added, and the mixture was stirred slowly for 1-2 min and allowed to stand for 1 min. Subsequently, 2 mL of no FCS-containing RPMI-1640 medium heated at 37°C was added slowly by taking 1 min, the mixture was allowed to stand for 1 min, furthermore, 4 mL of the medium was added again, the mixture was allowed to stand for 2 min, another 4 mL of the medium was added, and the mixture was allowed to stand for 4 min. Subsequently, 8 mL of RPMI-1640 medium containing 15% of FCS, 0.05 potency/L of streptomycin sulfate, 80,000 U of penicillin G potassium, 54 mg/L of gentamicin and 1 mL of pyruvate (called complete RPMI-

1640 medium) heated at 37°C was added slowly by taking 2-3 min, and the mixture was centrifuged at 500xg for 5 min. The supernatant was removed by suction, and the centrifugation cake was suspended in the complete RPMI-1640 medium heated at 37°C at a spleen cell density of 1 x 10^6 cells/mL. Subsequently, 0.1 mL each of the suspension was poured into a 96-well plate (Coater Co.), and the cultivation was carried out in an incubator set at 37°C, 5% CO_2 and 100% humidity for 24 h. Subsequently, 0.1 mL each of 10% FCS-added complete RPMI-1640 medium containing 1 x 10^{-4} M of hypoxanthine, 4 x 10^{-7} M of aminoputerine and 1.6 x 10^{-5} M of thymidine (called HAT medium, below) was added to each well. The supernatant was suctioned off on the 2nd day and 3rd day by 0.1 mL each, and 0.1 mL of the fresh HAT medium was added for exchange. Subsequently, the medium exchange procedures were carried out every 2-3 days. On the 6th day, the supernatant was removed by suction similarly, and the medium was changed to a complete RPMI-1640 medium containing 1 x 10^{-4} M of hypoxanthine and 1.6 x 10^{-5} M of thymidine (called HT medium, below). Subsequently, the proliferation was maintained by using the complete RPMI-1640 medium.

After the cell fusion procedures described above, the colonies became observable by naked eye in 10-14 days. When the cell occupied ¼ of the bottom area of the 96-well plate, the culture supernatant was tested by the enzyme-linked immunosorbent assay (ELISA) method using a human placenta-origin FN retaning ED-B as an antigen, and if the result was positive, the limiting dilution method [Method in Enzymology, 73, 3 (1981)] was immediately carried out for that well to carry out hybridoma cloning.

Specifically, 20 mL of a 10% FCS-added RPMI-1640 prepared to contain 1 x 10^8 cells of the Balb/c lineage mouse thymus gland cell was used, the hybridoma was sown in the amount of 0.2 mL each in a 6-well plate so that 3 cells/well, 1 cell/well and 0.3 cell/well to carry out cloning, and the desired hybridoma was established.

The above cloning was carried out by using the reactivity with cancer FN purified from the culture supernatant of WI-38VA13 cell, which is a normal human fibroblast WI-38 infected with a tumor virus SV40 or placenta-origin FN as an index. The above cloning procedures were repeated 4 times while confirming no reactivity with plasma FN, and as a result, 4 strains of hybridoma producing the monoclonal antibody of this invention having the desired reaction specificity were obtained.

Those strains were named OAL-TFN-01 – OAL-TFN-04.

The clones OAL-TFN-01–OAL-TFN-04 were cultivated in the complete RPMI medium at 37°C in the presence of 5% CO_2 for 96 h. The cultures were centrifuged at 3000 rpm for 10 min to obtain culture supernatants containing the desired monoclonal antibody.

Among those clones obtained, one strain (hybridoma OAL-TFN-01 producing the antibody of this invention) was selected.

The monoclonal antibody-producing cell was deposited as OAL-TFN-01 at the Agency of Industrial Science and Technology, Fermentation Research Institute with a deposit number of FERM P-11540. /15

The clone OAL-TFN-01, 1 x 10^6 cells was administered into the abdominal cavity of a Balb/c mouse inoculated with pristane (Aldrich) in advance. After 10-14 days, the ascitic fluid accumulated was collected to obtain a fluid containing the antibody of this invention.

The antibody in the ascitic fluid was purified by using gel chromatography (Sephacryl-S-300) and an ionic ion-exchange chromatography (Q-Sepharose) to obtain purified OAL-TFN-01 antibody.

The characteristics of the monoclonal antibody of this invention prepared as described above are shown in Application Example 3.

Application Example 3

Properties of the antibody of this invention

(1) Antibody subclass

The subclass of the monoclonal antibody of this invention was determined by using a mouse monoclonal antibody subclass identification kit (Bio-Rad).

As a result, the subclass of the antibody was found to be IgM.

(2) Antibody production level

The culture supernatant obtained in Application Example 2 was centrifuged, and the supernatant obtained was cultivated *in vitro* in a 10% FCS-added RPMI-1640 cultivation medium at 37°C in the presence of 5% CO_2 for 10 days.

When the hybridoma showed the maximum cell density, the amount of IgM of OAL-TFN-01 in the culture supernatant was about 5 μg/mL.

(3) Antibody potency

A 96-well polystyrene microplate (NUNC Co.) coated with 2 μg/well of purified placenta-origin FN holding ED-B (coating carried out at 4°C for 24 h) was blocked with a Dulbecco's phosphate buffer solution (pH 7.2, called D'PBS, below) of 1% BSA at 4°C for 24 h, subsequently, 50 μL of a culture supernatant containing the antibody of this invention and obtained in the Application Example 2 was added to each well of the plate, and the reaction was carried out at room temperature for 3 h. After washing 3 times with a washing buffer solution (D'PBS + 0.05% Tweren 20), the antibody bound to the ED-B-protein A fused protein of FN was measured by using a peroxidase-labeled goat anti-mouse immunoglobulin antibody (Zymet).

As a result, sufficient color development was observed with 1 x 10^1 time dilution of the culture supernatant.

(4) Standard curve by ELISA

The monoclonal antibody of this invention was diluted to 25 μg/mL by using D'PBS; 100 μL each was poured into each well of a 96-well microplate, the blocking was carried out overnight at 4°C and subsequently, the plate was washed with D'PBS (containing 0.05% Tweren 20). Subsequently, 300 μL of D'PBS, 0.05% thymerosal [transliteration] and 0.5% biome serum albumin (BSA) was added tto each well of the plate. The blocking was carried out overnight at 4°C. After blocking, the plate was washed with D'PBS (containing 0.05% of Tweren 20), and 100 μL of a 0.01 M phosphate buffer [0.1% NP-40 (NONIDENT P-40, Sigma), 0.05% thymerosal and 10% FCS, pH 5.5] was added to each well. Furthermore, 20 μL each of purified human plasma FN (pFN) and cancer FN (cFN) purified from culture supernatant of WI-38VA13 obtained by infecting normal human fibroblast WI-38 with a tumor virus SV40 at various concentrations were added, incubated at room temperature for 2.5 h and subsequently washed 6 times with D'PBS containing 0.05% of Tweren 20.

Furthermore, biotinylated anti-FN monoclonal antibody [OAL-pF115 established with /16
pFN of Sigma as an immunogen, see Rinsho Byouri, Vol. 35, Supplement, 1987, 119; The 18th Congress of the International Association of Medical Laboratory Technologists, Abstracts, p225 (1988), etc.] (x 1000 time diluted solution, 100 μL/well), D'PBS (100 μL/well), 0.1% CHAPS (3-[(3-chloramidepropyl)dimethylammonio]-1-propanesulfonate), 0.1% BSA, 0.05% thymerosal solution and 100 μL of buffer solution A were added to each well, the incubation was carried out for 2.5 h and washed 6 times with D'PBS containing 0.05% of Tweren 20.

Subsequently, 100 μL/well of avidin-peroxidase complex (Bio-Rad) dissolved in the buffer solution A was added, and the incubation was carried out for 1 h. After washing the plate with a washing buffer solution, 100 μL per well of o-phenylenediamine solution (OPD solution) was added, the reaction was carried out at room temperature for 10 min, subsequently, the reaction was stopped by adding 100 μL of 2 N sulfuric acid, and the absorbance at 492 nm was measured.

The above results are shown in Figure 1.

The ordinate is absorbance (OD) at 492 nm, and the abscissa is FN concentration; (1) shows the results of the cancer FN and (2) shows the results of plasma FN.

As is apparent from the results shown in the figure, the antibody of this invention is found not to react with plasma FN but dose-dependently with cancer FN.

Brief explanation of drawing

Figure 1 is a graph showing the results of the reactivity of the antibody of this invention with various kinds of FN.

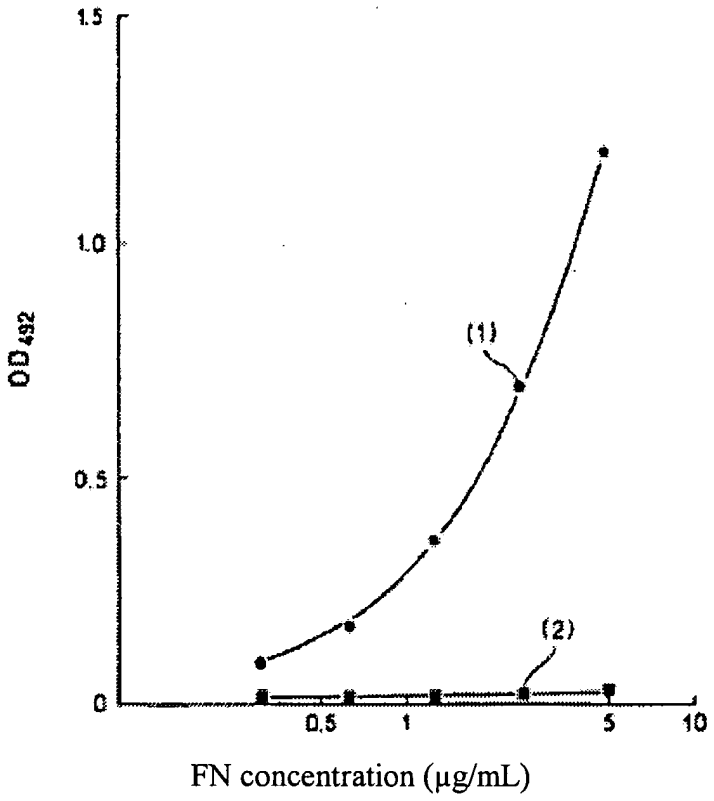

Figure 1